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Sources of PDGF expression in murine retina and the effect of short-term diabetes

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Purpose: Progressive dysfunction and death of vascular smooth muscle cells and pericytes is a pathophysiological hallmark of diabetic retinopathy, although the underlying mechanisms behind this process remain ill-defined. The multifunctional peptide platelet-derived growth factor (PDGF) is known to act as an important survival factor for both of these vascular cell-types at times of physiological stress. The retinal cell source(s) of PDGF remain unknown. It is important to understand how diabetes alters expression of this important growth factor.

Methods: Streptozotocin-diabetes was established in C57 mice. Following 8 weeks of sustained diabetes, the eyes were enucleated and in situ hybridization was used to localize expression of PDGF-A and PDGF-B chains in retina from both diabetic and non-diabetic controls. mRNA levels for both forms of PDGF, and their cognate PDGF-α and PDGF-β receptors, were also quantified using real-time PCR.

Results: In situ hybridization demonstrated that PDGF-A and PDGF-B were predominantly expressed by the retinal ganglion cells/nerve fibre layer in both normal and diabetic mice, and this localization pattern did not alter in diabetes. PDGF-A receptor was expressed exclusively in the ganglion cell layer of the retina while PDGF-B receptor was mostly localized to the Muller cell end-feet at the internal limiting membrane with lesser immunoreactivity in the ganglion cells, inner plexiform layer, and inner nuclear layer. PDGF-A and PDGF-α receptor mRNA expression levels remained unaltered between treatment groups, although retinal immunolocalization patterns between both receptors was distinct. However, there was a significant decrease of PDGF-B mRNA levels in diabetic retina when compared to non-diabetic controls ($p<0.001$), although there was no significant difference in PDGF-α receptor(insert space) expression.

Conclusions: Previous studies have shown PDGF expression in a range of cell-types during retinal development, but these results confirm ganglion cells as the principal PDGF source in mature retina. It may be significant that diabetes can reduce PDGF-B mRNA expression since this may have serious implications for vascular survival during diabetic retinopathy progression.

Platelet-derived growth factor (PDGF) is a multifunctional peptide that exists as two distinct isoforms (A and B chains) that can form homodimers or heterodimers and bind to specific α and β tyrosine kinase receptors [1]. PDGF is mitogenic to mesoderm-derived cells such as fibroblasts, vascular smooth muscle cells and chondrocytes, and is a potent chemoattractant and activator of neutrophils, monocytes, and fibroblasts in vitro [1]. In vivo, PDGF is an important modulator of wound healing and plays a critical pathogenic role in tumorigenesis, atherosclerosis, fibrosis, inflammatory disorders, and proliferative retinopathies [2]. During embryonic development, PDGF interacting with it's α or β receptor has an important role in organogenesis [3-5]. In homozygous PDGF-B null mice, there are fatal developmental abnormalities, and in the brain microvasculature, there is significantly reduced pericyte coverage and presence of microaneuryms [6]. PDGF-α receptor knockout mice are also severely deformed, with cranial malformations and deficiency in myotome formation [7], consistent with the fact that the α receptor binds both the A and B isoforms. Further studies on PDGF-A null mice suggest that this peptide plays a central role in modulation differentiation of progenitor cells into vascular smooth muscle cells [8].

In the retina, PDGF has an important role during health and disease. Particular focus has been placed on PDGF-B and its role as a potent survival factor for the retinal microvasculature in general and pericytes in particular [9]. Microvascular pericytes are the predominant cell type expressing PDGF-β receptors in the developing retina [10] and PDGF-B and the PDGF-β receptor have been shown to be crucial for the embryonic development and recruitment of pericytes by the developing vascular endothelium in the brain [6,11,12]. The A-chain of PDGF has been demonstrated in the adult retinal vasculature [13], although during development it appears only to be expressed by retinal neurons, and the cognate α-receptors by the adjacent astrocytes [14,15]. More recently, Hammes et al. [9] showed that when diabetes is induced in transgenic mice with only one functional PDGF-B allele, there is a greater loss of retinal pericytes and exacerbated capillary dropout in comparison to diabetic wild-type controls. Similar vascular pathology has been demonstrated in the renal glomerulus of neonatal mice treated with antibodies to the PDGF-β receptor;

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treated mice showed abnormal glomeruli with apoptotic endothelial cells and diminished numbers of mesangial cells [16]. In vitro experiments show that retinal microvascular endothelial cells modulate the proliferation of mural cell precursors through the expression of PDGF-B [17] and that isolated retinal vessels exposed to metabolic inhibitors are protected from death activation by PDGF-B [18].

The progressive dysfunction and depletion of the microvasculature is a pathogenic feature of diabetic retinopathy. There is also a widely recognized imbalance of growth factor gene expression and receptor activation that underlies hypervasopermeability, dysregulation of paracrine mediated cell communication, reduced cell survival and, eventually, inappropriate angiogenic responses driven by retinal hypoxia [19]. PDGF (both the A and B isoforms) and PDGF receptors have also been demonstrated to play a role in proliferative retinopathy and have been detected in retinal microvascular endothelial cells in postmortem retinal specimens [13]. Furthermore, both ligands and receptors have been shown to increase during proliferative retinopathy in epiretinal membranes and perhaps also in the retina proper [13]. Indeed, in transgenic mice that over express PDGF isoforms (in association with the rhodopsin promoter), there is enhanced retinal expression of PDGF-A leading to non-vascular associated proliferation of glial cells akin to proliferative vitreoretinopathy [20]. However, high expression of PDGF-B modulates proliferation of both vascular and nonvascular cells in a proliferative diabetic retinopathy-like fashion [20].

It is therefore important to identify sources of PDGF-A and PDGF-B in the retina and whether expression levels are altered during diabetes. As potent survival factors, it would be anticipated that alteration of PDGF expression, perhaps in unison with receptor expression, would have a major impact on retinal vascular function during hyperglycemia-mediated biochemical dysregulation. The current study examined PDGF-A and PDGF-B in parallel with α and β receptor expression to understand how these mRNAs are regulated in the retina during diabetes.

METHODS

Animal Model: Male c57Bl/6 mice weighing 20-25 g (5-6 weeks old) were randomly assigned to non-diabetic control or diabetic groups. Diabetes was induced by a single intra-peritoneal injection of streptozotocin (Sigma) at 180 mg/Kg body weight [21]. Control animals received an equivalent dose of the drug vehicle (citrate buffer at pH 4.6). The mice were caged individually and allowed food and water ad lib. Blood glucose levels were measured fortnightly. Diabetic animals with blood glucose levels between 20 and 30 mM were included in the study. Groups of 8-10 animals were taken for each experimental and control group and the experiment was carried out 3 times. All animals were sacrificed after an 8 week duration of diabetes. One eye from each mouse was processed for RNA extraction for PCR studies, whilst the other was processed for in situ hybridization. The former were pooled and snap frozen in liquid nitrogen. The latter were fixed immediately in freshly prepared 4% paraformaldehyde (PFA) for 4 h at room temperature.

Quantification of mRNAs: Freshly dissected retinas (at least 6-8 retinas per sample; three samples per experimental group were subsequently amplified by RT-PCR) from both non-diabetic and diabetic mice were snap-frozen in liquid nitrogen. These retinas were pooled and RNA was extracted using the RNeasy Mini Kit (Qiagen, Crawley, UK). The quantity of RNA in each sample was determined spectrophotometrically (U 1100 model, Hitachi Europe Ltd., Berkshire, UK) and the purity and quality of each RNA sample was estimated by visualisation of clear 18S and 28S ribosomal RNA bands after electrophoresing 1 μg of each sample on a 1% agarose gel.

The RNA from each extraction was reverse transcribed into cDNA using a first Strand cDNA Synthesis Kit (Life Technologies, Paisley, UK) and random hexamer primers (Boehringer Mannheim, Mannheim, Germany). Real-time PCR was conducted for quantitative analysis of mRNA expression using sequence-specific primers for PDGF-A (Forward: 5'-GTC CAG GTG AGG TTA GAG G-3', Reverse: 5'-CAC GGA GGA GAA CAA AGA C-3', giving a 210 bp fragment); PDGF-B (Forward 5'-TGA AAT GCT GAG CAC CAC-3', Reverse: 5'-AGC TTT CCA ACT CGA CTC C-3', giving a 137 bp fragment); PDGF-α (Forward: 5'-CAA ACC CTG AGA CCA CAA TG-3', Reverse: 5'-TCC CCC AAC AGT AAT CCA AG-3', giving a 235 bp fragment); PDGF-β (Forward: 5'-TGC CTC AGC CAA ATG TCA CC-3', Reverse: 5'-TGC TCA CCA CCT CGT ATT CC-3', giving a 159 bp fragment). Primers to amplify the housekeeping gene acidic ribosomal phosphoprotein (ARP) were also used: (Forward: 5'-CGA CCT GGA AGT CCA ACT AC-3', Reverse: 5'-ATC TGC TGC ATC TGC TTG-3', giving a 109 bp fragment).

Real-time PCR was performed using a LightCycler rapid thermal cycler system (Roche Hertfordshire, UK) according to protocols outlined by Simpson et al. [22]. The PCR reaction was performed in glass capillary reaction vessels (Roche) in a 20 μl volume with 0.5 μM primers. Reaction buffer, 2.5 mM $MgCl_2$, dNTPs, Hotstart *Taq* DNA polymerase and SYBR Green I were included in the QuantitTect LightCycler-SYBR Green PCR Master Mix (Qiagen). Amplification of cDNAs involved a 15 min denaturation step followed by 40 cycles with a 95 °C denaturation for 15 s, 55-58 °C annealing for 20 s and 72 °C for an appropriate extension time (5-25 s). Fluorescence from SYBR Green I bound to the PCR product was detected at the end of each 72 °C extension period. The specificity of the amplification reactions was confirmed by melting curve analysis and subsequently by agarose gel electrophoresis [22]. The quantification data were analysed with the LightCycler analysis software as described previously [22]. The baseline of each reaction was equalized by calculating the mean value of the five lowest measured data points for each sample and subtracting this from each reading point. Background fluorescence was removed by setting a noise band. The number of cycles at which the best-fit line through the log-linear portion of each amplification curve intersects the

noise band is inversely proportional to the log of copy number. A dilution series of a reference cDNA sample was used to generate a standard curve against which the experimental samples were quantified. For each gene, PCR amplifications were performed in triplicate on at least two independent RT reactions. Statistical analysis was performed between the results obtained from the normal versus the diabetic retina using a paired Student's t-test (two tailed).

In situ hybridization: Fixed eyes were washed in PBS and then dehydrated in a graded alcohol series. They were then cleared in toluene and individually embedded in paraffin wax for standard histological sectioning (5 μm sections) on silane-coated slides. Prior to in situ hybridization, riboprobes were prepared from PCR products derived from murine PDGF-A and PDGF-B retinal RNA. Riboprobes were prepared as previously described [23]. Briefly, the PCR product (produced as outlined above) were cloned into the pGEM-T vector (Promega) and its identity and orientation confirmed by restriction analysis (ABI Prism377 DNA Sequencer). The recombinant plasmid was then linearised to provide templates for transcription of sense and antisense riboprobes with either T7 or SP6 RNA polymerase, incorporating digoxygenin dUTP (Boehringer Mannheim).

In situ hybridization was performed according to a protocol outlined previously for paraffin wax sections of retina [23,24]. Briefly, the sections were de-waxed, re-hydrated and post-fixed in 4% PFA. Following fixation the sections were washed in PBS and the proteins denatured in 200 mM HCl for 10 min. The sections were then digested with proteinase K (20 μg/ml in PBS/50 mM EDTA) for 30 min at 37 °C followed by washing in PBS. The riboprobes were hybridized to the sections (about 20 ng/100 μl buffer) for 18 h at 42 °C in a saline sodium citrate (SSC) hybridization buffer consisting of 10% dextran sulphate, 10 mM dithiothreitol (DTT), 0.02% sodium dodecyl sulphate (SDS), 50% formamide and 10 mg/ml salmon sperm DNA. Following hybridization, the sections were washed in descending SSC solutions at room temperature and placed in PBS. Anti-digoxygenin alkaline phosphatase antibody (Boehringer Mannheim Ltd.) was then added for 2 h followed by washing in PBS. Hybridized probes were detected using nitroblue tetrazolium solution (NBT, 75 mg/ml in dimethylformamide; Boehringer Mannheim Ltd.) dissolved in a Tris buffer (100 mM, pH 9.5) containing NaCl (100 mM), $MgCl_2$ (50 mM) and levamisole (2 mg/ml). Sections were counterstained with 0.02% fast green, washed and mounted with Glycermount (Dako Ltd., Cambridgeshire, UK).

Immunohistochemistry: Sections of mouse eyes were dewaxed and rehydrated in PBS. The sections were then subjected to antigen retrieval for 20 min in citrate buffer (pH 6.0) in a pressure cooker. After washing in PBS, the sections were blocked with 5% normal goat serum (NGS), 1% BSA, 0.01% Triton-X100 and then incubated in primary antibodies to either PDGF-α receptor or PDGF-β receptor (Santa Cruz Inc., CA, USA) at 1:100 dilution overnight at 4 °C. Controls were perfomed using primary antibody exclusion and rabbit non-immune serum. Prior to detection using the anti-rabbit Envision+System (Dako Ltd.), endogenous peroxidase activity was quenched in 3% hydrogen peroxide. After allowing diaminobenzidine (DAB) reaction product to develop, the sections were then washed extensively, counterstained with haemotoxylin, and mounted with Glycermount (Dako Ltd.).

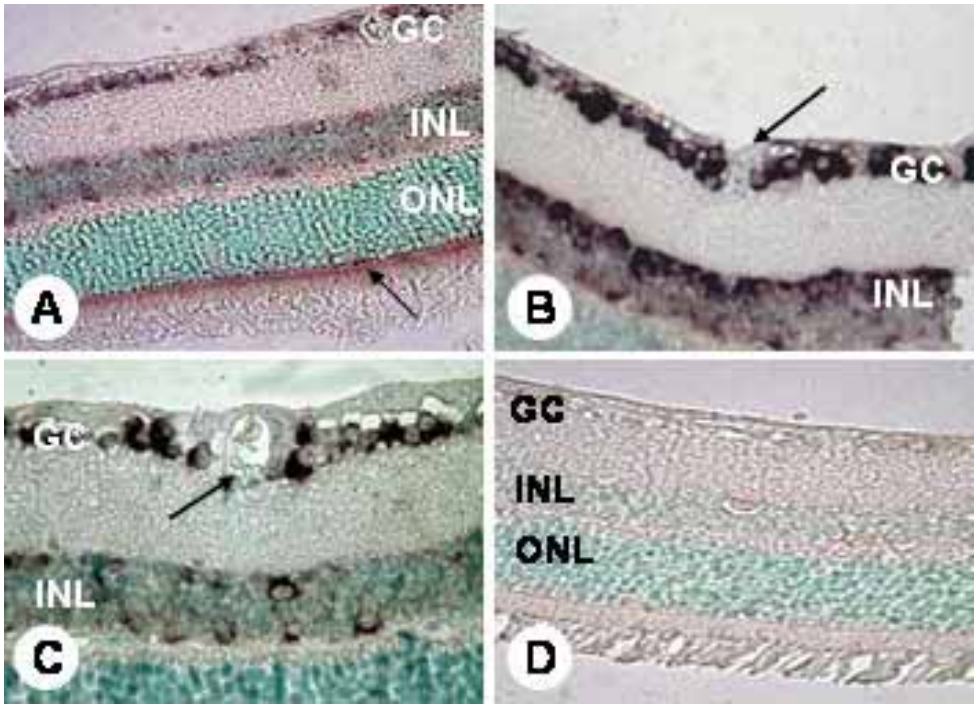

Figure 1. Localization of PDGF-A mRNA expression. In situ hybridization in retinal sections from non-diaebtic (**A** and **B**) and diabetic mice (**C**) with PDGF-A riboprobes shows mRNA expression in the inner retina, specifically localized to the ganglion cell layer (GC) and both the inner and outer aspects of the inner nuclear layer (INL). In the outer retina there is expression localized to the inner segments of the photoreceptor cells (**A**; arrow). When retinas from non-diabetic and diabetic mice are compared, there are no obvious differences in reaction product localization or intensity (compare **B** with **C**). It is notable that the retinal blood vessels (**B**, **C**; arrows) show no expression of PDGF-A. Negative hybridization with sense riboprobe is shown in **D**. Original magnifications: x400 (**A**, **D**) and x100 (**B**, **C**). The outer nuclear layer (ONL) is also labeled in the micrograph.

RESULTS

Characterization of diabetes: STZ-treated mice displayed cessation of growth but without significant weight loss over the 8 week period. Once diabetes was established, the animals exhibited polyuria/polydipsia and fed blood-glucose levels of 15-30 mM/l. However, only animals with fed blood-glucose measurements of between 20 and 30 mM/l were included in the study (mean blood glucose 26.97±3.6 mM/l). Both fed and fasting blood glucose measurements were obtained from control mice and were 8.12±1.26 mM/l and 3.79±0.33 mM/l, respectively. Control animals with fed blood glucose measurements greater than 10 mM/l were excluded from the study.

Localization of PDGF-A/PDGF-B sources in retina: In situ hybridization illustrated the presence of both PDGF-A and PDGF-B gene expression as a deep blue-purple NBT/BCIP alkaline phosphatase reaction product within both control and diabetic mouse retinas. The most intense staining for PDGF-A and PDGF-B mRNA was localized within the cell bodies of the retinal neurons and both probes showed the same pattern in terms of the different cell types stained and the relative staining intensity (Figure 1 and Figure 2). Although the retinal ganglion cells were particularly prominent, significant expression of both PDGF-A and PDGF-B was indicated within the inner nuclear layer (INL) of the retina and in two distinct sub-populations of cells in particular, located at the inner and outer aspects of the INL (Figure 1A and Figure 2A). These two subpopulations corresponded to horizontal cells at the outer aspect of the INL and amacrine cells at the inner (Figure 1C,D). Significant positive staining was also present within the inner segments of the photoreceptor cells.

The pattern of expression of both the PDGF isoforms appeared identical in both diabetic and control mice; the same types of cells that stained positively in the control retina also proved positive in the diabetic retina (compare panels A and B with panels C and D in Figure 1 and Figure 2).

The retinal vasculature showed no evidence of PDGF-A and PDGF-B expression in either diabetic or control retina (Figure 1B,D and Figure 2B,D). Indeed the vascular cells provided the most negatively stained structures throughout these preparations.

Weak, non-specific staining indicated non-binding of the control “sense” probes and there was no obvious difference in the level of staining between samples from control mice and diabetics.

PDGF-Receptor Immunolocalization: PDGF-α immunoreactivity was localized almost exclusively to the ganglion cell layer of the retina, with extensive reaction product being detected in the axons of these cells in the nerve fiber layer (Figure 3). Axonal immunoreactivity to PDGF-α was especially intense at the region of the optic nerve head where the ganglion cell axons coalesced (Figure 3C). There was no apparent difference between diabetic and non-diabetic retina.

The pattern of PDGF-β receptor immunoreactivity was distinct from that observed for the PDGF-α receptor (Figure 4). PDGF-β was mostly localized to the Muller cell end-feet at the internal limiting membrane with lesser immunoreactivity in the ganglion cells, inner plexiform layer and inner nuclear layer (Figure 4). There was no difference between diabetic and non-diabetic retina.

PDGF and PDGF-receptor expression: The overall results pooled from representative PCR reactions are shown in Figure 5. In order to procure relative values for the gene expression, PCR reactions were carried out for ARP, which is a housekeeping gene whose expression was not considered to be vulnerable to diabetes-induced alterations [22]. The data collected for the PDGF genes was then normalized to the values obtained for ARP, in order to compare relative expression between the groups (Figure 5). There was a significant de-

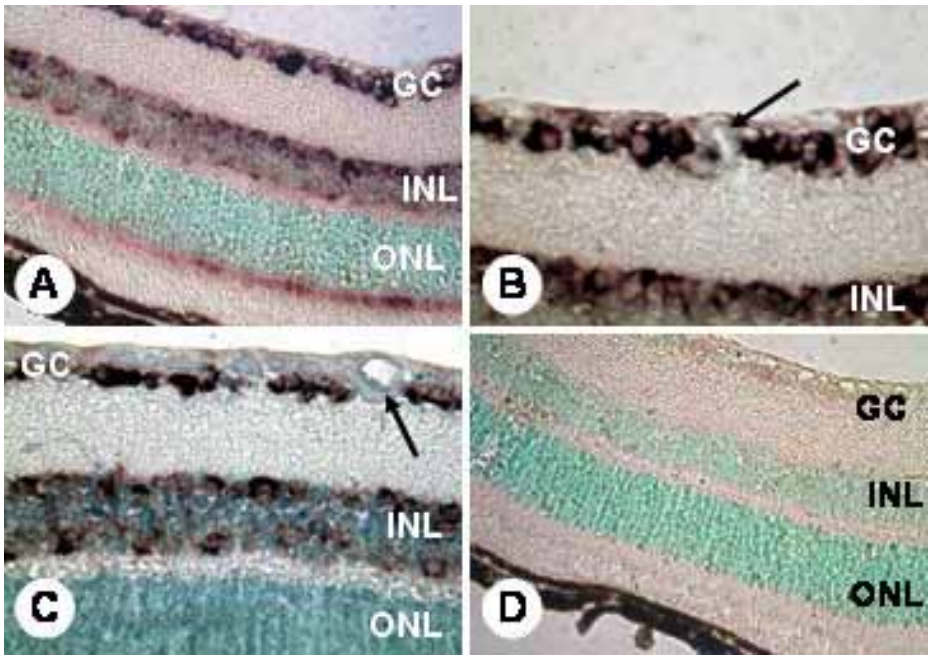

Figure 2. Localization of PDGF-B mRNA expression. Hybridization with PDGF-B riboprobes shows a largely similar localization pattern to that observed with PDGF-A (see Figure 1). Non-diabetic (**A**, **B**) and diabetic murine retina (**C**) shows mRNA expression in the ganglion cell layer (GC), the innermost aspect of the inner nuclear layer (INL) and to a small, extent in the inner segments of the photoreceptors (**A**). When retinas from non-diabetic and diabetic mice are compared, there are no obvious differences in reaction product localization or intensity (compare **B** with **C**) and there is no PDGF-B mRNA in the retinal blood vessels (**B**, **C**; arrows). Sense riboprobes showed no localization of mRNAs (**D**). Original magnifications: x400 (**A**, **D**) and x100 (**B** and **C**). The outer nuclear layer (ONL) is also labeled in the micrograph.

crease in PDGF-B mRNA expression in diabetic retina when compared to non-diabetic controls ($p<0.001$; Figure 5). There was no significant alteration in the levels of either PDGF-A or either of the PDGF receptors (Figure 5). There was a slight increase in PDGF-α expression but this was not statistically significant.

DISCUSSION

This study demonstrated retinal sources of PDGF and PDGF-receptors. Previously, PDGF was known to be expressed by several retinal cell types during development [14], but until recently had only been identified in the vasculature of mature retina [13]. The present study demonstrated mRNA expression for PDGF-A and PDGF-B within ganglion cells of adult mice, and also within discrete populations of neurons at the inner and outer aspects of the inner nuclear layer. There was also consistent localization of mRNA for both PDGF isoforms within the inner segments of the photoreceptor cells.

As PDGF functions principally as a paracrine or autocrine growth factor within tissues, the spatial locations of the cells expressing it may be of relevance. PDGF is not only of importance in pericyte recruitment and survival, but has been shown

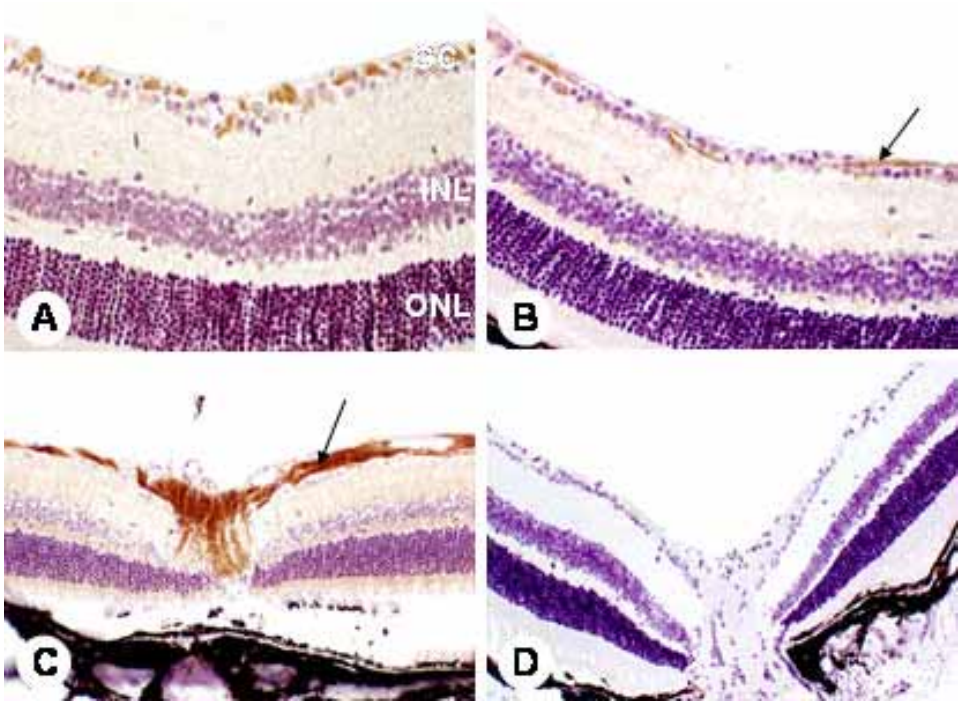

Figure 3. Immunohistochemistry of PDGF-α. Immunohistochemistry of PDGF-α in retinas of diabetic and non-diabetic mice. The retinal ganglion cells (GC) and nerve fiber layer (arrow) of both non-diabetic (**A**) and diabetic (**B**) show strong immunoreactivity. The axonal concentration of this receptor (far removed from the cell body) is exemplified by strong PDGF-α immunreactivity at the optic nerve head where ganglion cell axons coalesce (**C**, arrow). Controls show no apparent deposition of DAB reaction product (**D**). Original magnifications: x200 (**A**, **B**); x100 (**C**, **D**). The outer nuclear layer (ONL) and the inner nuclear layer (INL) are also labeled in the micrograph.

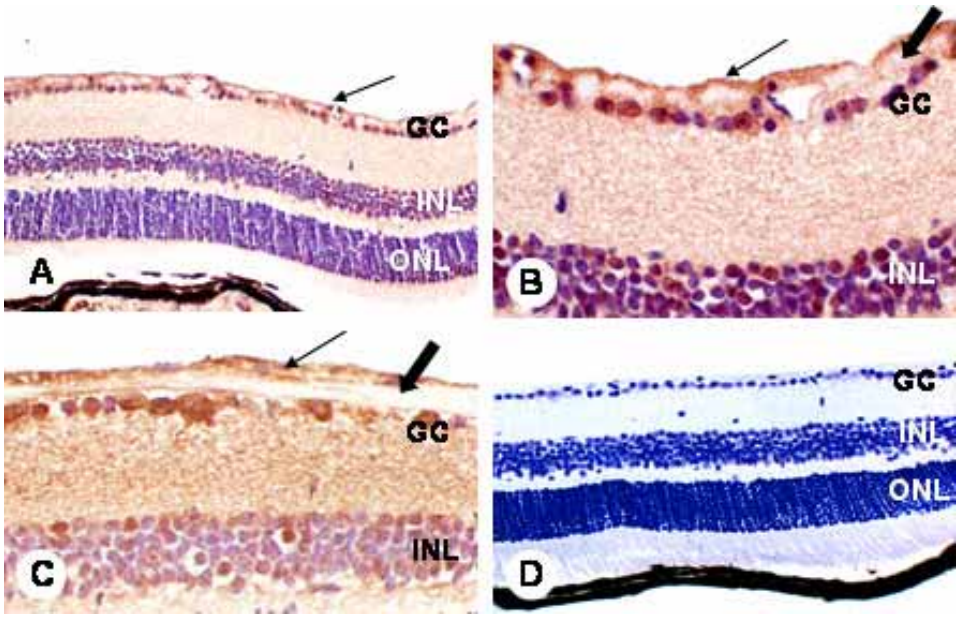

Figure 4. Immunohistochemistry of PDGF-β. Immunohistochemistry of PDGF-β in retinas of diabetic and non-diabetic mice. The Muller cell end-feet at the internal limiting membrane demonstrate most intense immunoreactivity (small arrow; **A**-**C**). Lesser DAB reaction product is apparent in the retinal ganglion cells (GC) and inner nuclear layers (INL; **A**, **B**) but there is no immunoreactivity in the nerve fiber layer (large arrow) in contrast to PDGF-α localization. There are no clear differences in intensity and staining pattern between non-diabetic (**A**, **B**) and diabetic retinas (**C**). Controls showed no immunoreactivity (**D**). Original magnifications: x200 (**A**, **D**); x400 (**B**, **C**). The outer nuclear layer (ONL) is also labeled in the micrograph.

to control migration of retinal astrocytes from the optic nerve, and to be responsible for astrocyte proliferation in transgenic mice over expressing the PDGF-A isoform [25]. In this regard it is notable that the astroglia within the mouse retina are close to the vitreal interface and juxtaposed to the ganglion cells. The ganglion cells are also perfectly located to act as a source of PDGF for the vascular smooth muscle cells of the major retinal vessels and the pericytes of the capillaries in the nerve fiber layer of the central retina. Likewise, the locations of PDGF expressing neurons at the inner and outer aspects of the inner nuclear layer coincide with the two major capillary beds of the inner retina and may serve as rich sources of PDGF for the local mural cells.

The demonstration of PDGF expression by the photoreceptor cells in the present study may have important implications for cell survival and cellular interactions at the outer retina. An immunohistochemical study has shown no evidence of PDGF receptors in either photoreceptors or retinal pigment epithelial (RPE) cells [13]. However PDGF has been shown to act as a paracrine growth factor for RPE cells in culture [26] and has important roles in proliferative vitreo-retinopathy, stimulating RPE proliferation and chemotaxis [27], and mediating contraction of the epiretinal membranes that produce wrinkling and detachment of the neural retina in this condition [28]. Therefore, as PDGF has obvious roles in RPE cell growth promotion and kinetics under pathological conditions, it is possible that it may have maintenance or survival functions in these cells under normal circumstances. The localization of PDGF mRNA expression to the photoreceptor cells in the current investigation would be consistent for such a role for PDGF in the RPE function.

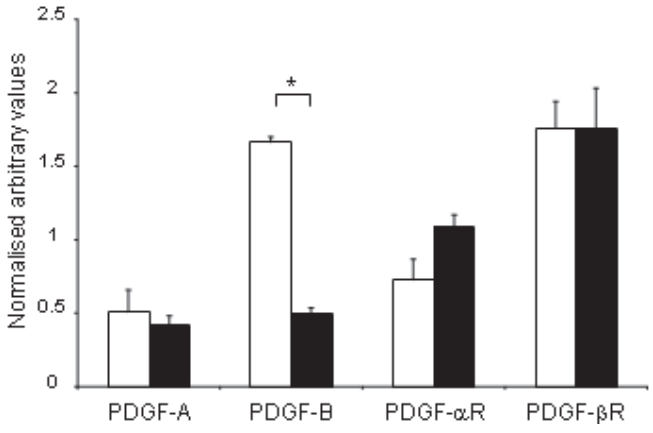

Figure 5. Quantification of PDGF peptide and receptor mRNAs.. Real-time RT-PCR was conducted on PDGF-A, PDGF-B, PDGF-α receptor and PDGF-β receptor mRNAs in normal and diabetic murine retina. When retinal extracts from non-diabetic controls (white bars) were compared with those from diabetics (black bars) there was a significant decrease in PDGF-B mRNA expression the diabetic retina (*p<0.001). PDGF-α receptor mRNA showed a small increase in diabetic retina but this was not statistically significant. The graph shows normalized values against the housekeeping gene ARP (Acidic ribosomal phosphoprotein PO). Both PDGF-A and PDGF-β receptor mRNAs were unaltered between mouse groups.

The lack of expression of PDGF-A and PDGF-B mRNA in retinal vascular cells in the present study was surprising, as a previous immunohistochemical study had suggested that PDGF immunoreactivity in the normal retina was only associated with the vasculature [13]. However, it is possible that the growth factor is expressed by the neurons and localizes in the vascular basement membranes by virtue of its heparin-binding properties. Alternatively, as the study by Robbins et al. [13] employed post-mortem human retina, which normally involves delayed fixation, it is also possible that the PDGF localized in the retinal vessels represented aberrant PDGF expression by vascular endothelial cells during post-mortem hypoxia. Increased expression of PDGF has been noted previously by vascular endothelial cells during hypoxia [29] and retinal vascular endothelial cells remain viable for many hours in post mortem retina (they may be cultured up to 48 h post-mortem). On this issue it is notable that a later study using well fixed experimental porcine retina demonstrated PDGF immunoreactivity throughout the tissue but mentioned no specific association with the normal retinal vessels [30]. In common with PDGF expression in the present study, previous in situ hybridization studies have shown that other vasogenic growth factors are predominantly expressed by the retinal neurons [23,24,31].

In the current study, quantitative real-time PCR investigation revealed a significant reduction in the expression of PDGF-B in the retina of diabetic animals when compared to non-diabetic controls. mRNA levels of PDGF-A, PDGF-α, and PDGF-β were not significantly altered between groups. This was also borne-out by PDGF-receptor immunohistochemistry evaluation, although the cell-staining pattern of both receptors was distinct, with PDGF-α being largely localized to ganglion cells and PDGF-β being observed extensively in the Muller glia. The mechanism for the marked decrease in PDGF-B mRNA expression during short-term diabetes remains unclear, however, it would seem likely that this would translate to an equivalent drop in the availability of the PDGF-B peptide. This could have serious consequences for several retinal cells, especially retinal pericytes, which depend on PDGF as a survival factor [9,18] and could significantly exacerbate the vasodegenerative pathology during diabetic retinopathy. It may be expected that dependent cells would try to compensate for a lack of PDGF-B by increased expression of their PDGF receptors, which, as PDGF-B can bind to both the α and β subunits, could include both forms of the PDGF receptor. In the present study this appears to have occurred to some extent in the case of PDGF-α.

A reduction in PDGF-B expression in diabetes reveals yet another inequality between the vascular endothelial cells and pericytes of the retinal microvasculature during diabetes. Diabetes has been shown to up-regulate the expression of vascular endothelial growth factor in the retina [19,32], a major survival factor for endothelial cells, but increased PDGF has only been demonstrated in proliferative fibrovascular tissue from epiretinal membranes [32] removed from patients with end-stage proliferative diabetic retinopathy or in vitreous from

similar patients where the growth factor may have been derived from haemorrhage or vascular leakage [33]. Therefore, in early diabetes, endothelial cells may be sustained while pericytes are deprived of their principal growth factor. This inequality may contribute to the differential loss of pericytes as compared to endothelial cells in diabetic retinopathy.

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REFERENCES

1. Heldin CH, Ostman A, Ronnstrand L. Signal transduction via platelet-derived growth factor receptors. Biochim Biophys Acta 1998; 1378:F79-113.
2. Rosenkranz S, Kazlauskas A. Evidence for distinct signaling properties and biological responses induced by the PDGF receptor alpha and beta subtypes. Growth Factors 1999; 16:201-16.
3. Lipschutz JH. Molecular development of the kidney: a review of the results of gene disruption studies. Am J Kidney Dis 1998; 31:383-97.
4. Heldin CH, Westermark B. Mechanism of action and in vivo role of platelet-derived growth factor. Physiol Rev 1999; 79:1283-316.
5. Bostrom H, Gritli-Linde A, Betsholtz C. PDGF-A/PDGF alpha-receptor signaling is required for lung growth and the formation of alveoli but not for early lung branching morphogenesis. Dev Dyn 2002; 223:155-62.
6. Lindahl P, Johansson BR, Leveen P, Betsholtz C. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. Science 1997; 277:242-5.
7. Soriano P. The PDGF alpha receptor is required for neural crest cell development and for normal patterning of the somites. Development 1997; 124:2691-700.
8. Lindahl P, Karlsson L, Hellstrom M, Gebre-Medhin S, Willetts K, Heath JK, Betsholtz C. Alveogenesis failure in PDGF-A-deficient mice is coupled to lack of distal spreading of alveolar smooth muscle cell progenitors during lung development. Development 1997; 124:3943-53.
9. Hammes HP, Lin J, Renner O, Shani M, Lundqvist A, Betsholtz C, Brownlee M, Deutsch U. Pericytes and the pathogenesis of diabetic retinopathy. Diabetes 2002; 51:3107-12.
10. Benjamin LE, Hemo I, Keshet E. A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. Development 1998; 125:1591-8.
11. Crosby JR, Seifert RA, Soriano P, Bowen-Pope DF. Chimaeric analysis reveals role of Pdgf receptors in all muscle lineages. Nat Genet 1998; 18:385-8.
12. Hellstrom M, Kalen M, Lindahl P, Abramsson A, Betsholtz C. Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. Development 1999; 126:3047-55.
13. Robbins SG, Mixon RN, Wilson DJ, Hart CE, Robertson JE, Westra I, Planck SR, Rosenbaum JT. Platelet-derived growth factor ligands and receptors immunolocalized in proliferative retinal diseases [published erratum in Invest Ophthalmol Vis Sci 1995; 36:519]. Invest Ophthalmol Vis Sci 1994; 35:3649-63.
14. Mudhar HS, Pollock RA, Wang C, Stiles CD, Richardson WD. PDGF and its receptors in the developing rodent retina and optic nerve. Development 1993; 118:539-52.
15. Fruttiger M, Calver AR, Kruger WH, Mudhar HS, Michalovich D, Takakura N, Nishikawa S, Richardson WD. PDGF mediates a neuron-astrocyte interaction in the developing retina. Neuron 1996; 17:1117-31.
16. Sano H, Ueda Y, Takakura N, Takemura G, Doi T, Kataoka H, Murayama T, Xu Y, Sudo T, Nishikawa S, Nishikawa S, Fujiwara H, Kita T, Yokode M. Blockade of platelet-derived growth factor receptor-beta pathway induces apoptosis of vascular endothelial cells and disrupts glomerular capillary formation in neonatal mice. Am J Pathol 2002; 161:135-43.
17. Hirschi KK, Rohovsky SA, Beck LH, Smith SR, D'Amore PA. Endothelial cells modulate the proliferation of mural cell precursors via platelet-derived growth factor-BB and heterotypic cell contact. Circ Res 1999; 84:298-305.
18. Kodama T, Oku H, Kawamura H, Sakagami K, Puro DG. Platelet-derived growth factor-BB: a survival factor for the retinal microvasculature during periods of metabolic compromise. Curr Eye Res 2001; 23:93-7.
19. Cai J, Boulton M. The pathogenesis of diabetic retinopathy: old concepts and new questions. Eye 2002; 16:242-60.
20. Mori K, Gehlbach P, Ando A, Dyer G, Lipinsky E, Chaudhry AG, Hackett SF, Campochiaro PA. Retina-specific expression of PDGF-B versus PDGF-A: vascular versus nonvascular proliferative retinopathy. Invest Ophthalmol Vis Sci 2002; 43:2001-6.
21. Cuthbertson RA, and Mandel TE. Pancreatic islet transplantation ameliorates the effect of diabetes on retinal capillary endothelial cell kinetics. Curr Eye Res 1989; 8: 681-6.
22. Simpson DA, Feeney S, Boyle C, Stitt AW. Retinal VEGF mRNA measured by SYBR green I fluorescence: a versatile approach to quantitative PCR. Mol Vis 2000; 6:178-83 .
23. Simpson DA, Murphy GM, Bhaduri T, Gardiner TA, Archer DB, Stitt AW. Expression of the VEGF gene family during retinal vaso-obliteration and hypoxia. Biochem Biophys Res Commun 1999; 262:333-40.
24. Stitt AW, Simpson DA, Boocock C, Gardiner TA, Murphy GM, Archer DB. Expression of vascular endothelial growth factor (VEGF) and its receptors is regulated in eyes with intra-ocular tumours. J Pathol 1998; 186:306-12.
25. Yamada H, Yamada E, Ando A, Seo MS, Esumi N, Okamoto N, Vinores M, LaRochelle W, Zack DJ, Campochiaro PA. Platelet-derived growth factor-A-induced retinal gliosis protects against ischemic retinopathy. Am J Pathol 2000; 156:477-87.
26. Campochiaro PA, Hackett SF, Vinores SA, Freund J, Csaky C, LaRochelle W, Henderer J, Johnson M, Rodriguez IR, Friedman Z, et al. Platelet-derived growth factor is an autocrine growth stimulator in retinal pigmented epithelial cells. J Cell Sci 1994; 107:2459-69.
27. Vinores SA, Henderer JD, Mahlow J, Chiu C, Derevjanik NL, Larochelle W, Csaky C, Campochiaro PA. Isoforms of platelet-derived growth factor and its receptors in epiretinal membranes: immunolocalization to retinal pigmented epithelial cells. Exp Eye Res 1995; 60:607-19.
28. Carrington L, McLeod D, Boulton M. IL-10 and antibodies to TGF-beta2 and PDGF inhibit RPE-mediated retinal contraction. Invest Ophthalmol Vis Sci 2000; 41:1210-6.
29. Kourembanas S, Hannan RL, Faller DV. Oxygen tension regulates the expression of the platelet-derived growth factor-B chain gene in human endothelial cells. J Clin Invest 19990; 86:670-4.

30. Xiao M, McLeod D, Cranley J, Williams G, Boulton M. Growth factor staining patterns in the pig retina following retinal laser photocoagulation. Br J Ophthalmol 1999; 83:728-36.
31. Gerhardinger C, Brown LF, Roy S, Mizutani M, Zucker CL, Lorenzi M. Expression of vascular endothelial growth factor in the human retina and in nonproliferative diabetic retinopathy. Am J Pathol 1998; 152:1453-62.
32. Paques M, Massin P, Gaudric A. Growth factors and diabetic retinopathy. Diabetes Metab 1997; 23:125-30.
33. Freyberger H, Brocker M, Yakut H, Hammer J, Effert R, Schifferdecker E, Schatz H, Derwahl M. Increased levels of platelet-derived growth factor in vitreous fluid of patients with proliferative diabetic retinopathy. Exp Clin Endocrinol Diabetes 2000; 108:106-9.

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